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The origin of novel trait inferred from transcriptomic analysis and a targeted gene approach in the beetle horns.

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The origin of novel trait inferred from transcriptomic analysis and a targeted gene approach in the beetle horns.

Naureen Fatima

*Thesis submitted
to the Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements
for the degree of Master of Science in
Genetics and Developmental Biology*

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ABSTRACT

The origin of novel trait inferred from transcriptomic analysis and a targeted gene approach in the beetle horns.

Naureen Fatima

The origin of the evolutionary new trait (evolutionary novelty) and its subsequent evolution is of great interest for biologists in various fields, and beetle horns have been used to address this fundamental biological question. Previous studies on one of the horned beetle species, *Onthophagus taurus*, that utilized comparative gene expression analyses, suggested legs to be a strong candidate of the origin of horns. At the same time, their horns are secondary sexual traits whose development is regulated by the same gene (*doublesex*) as genitalia, which also originates from paired appendages such as legs. However, little is known about the similarity or difference in the gene expression pattern between horns and genitalia. At the same time, the horn shows an extreme form of plasticity influenced by nutrition uptake in males of *O. taurus* (polyphenism). This polyphenic status might have been acquired when (or after) the horn was invented. However, it is unclear if the same set of genes indicate sensitivity to nutritional input during the development of legs, genitalia, and horns. To address these questions, we utilized RNAseq with four tissues. We highlighted the gene expression pattern in polyphenic head horns (where nutritional conditions drastically affect its size), non-polyphenic thoracic horns (where nutritional conditions have moderate effects on size), non-horn appendages, legs and genitalia (where nutritional conditions do not affect the size) in two beetle developmental stages, prepupa and pupa.

Our transcriptomic data indicated a clear difference between the two developmental stages (pupa and prepupa). Furthermore, Spearman's correlation showed less correlation between horns and genitalia, inferring the close relatedness of horns and legs more than genitalia. We also detected more differentially expressed (D.E.) genes between horns and genitalia than legs, suggesting legs to be still the most likely candidate of the origin of horns. Our transcriptomic data provided us with a list of genes. Among many, we detected genes that are involved in the wingless signaling pathway such as Wnt-1 (*Wingless* in *Drosophila*), *frizzled-2*, *frizzled-4*, and low-density lipoprotein receptor-related protein 6 (*arrow* in *Drosophila*) whose developmental function in the horn is not studied well. Wg pathway is known to interact with Hedgehog pathway that is also known to be involved in both horn development and polyphenism. Thus, we took a targeted gene approach where RNAi was utilized to knockdown the gene *arrow* to examine its horn developmental role. Our data suggest that *arrow* inhibits horn development in a dosage-dependent manner. Our results also showed the effects of *arr* knockdown in other tissues such as wings and legs. Thus, my research successfully integrated transcriptomic study and targeted gene approach to shed light further into the genetic underpinnings of the origin and evolution of the novel trait, beetle horns.

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Introduction:

The origin and evolution of novel traits are among the most elementary yet inconclusive topics in evolutionary biology. Novel traits, as the name indicates, are the newly acquired traits that lack obvious homology to any structure in their ancestral species and also within the same organism (1). An excellent example of novel trait includes the wing patterns of butterflies, which often serve as protection against predators and are unique to Lepidopterans. Another classic example of novel trait includes beetle horns that are very diverse in their morphology. Number of studies in the past are committed in understanding the biological function of this structure. Originally, beetle horns were proposed to be used by the males only to seize their female mating partners (2) or as protection against predators (3). Some scientists suggested horns as digging tools used by the beetles and later co-opted for fighting their rivals (4), whereas others suggested horns as digging tools for gashing plants to feed on their sap (5). Some even suggested it to be a mere extension of the large body part and proposed horns to be functionless (6). Finally, it was accepted as weapons used by males against their rivals to gain access to their female mating partners (7). The use of horns as weapons in battle has been confirmed in various species.

The environment-responsive trait formation has always sought attention from evolutionary and developmental biologists (8,9). Phenotypic plasticity is defined as the potential of a single genotype to produce different phenotypes due to variation in response to specific environmental conditions (10,11). While phenotypic plasticity has a linear relationship with environmental factors, a special case of phenotypic plasticity known as polyphenism produces two or more distinct morphs. A classic example

includes *Nemoria arizonaria* caterpillars, which have two seasonal morphs: the summer seasonal form mimics an oak catkin when fed on oak flowers. The winter to early spring form mimics an oak twig when fed on oak leaves (11).

The horns of *Onthophagus*

Figure 1: Scaling relationship between body size and horn length in *O. taurus*. Body size has a linear relationship with larval food quality and quantity. Horn size has a bimodal relationship with body size and thus develop into one of two extreme morphs. They develop either as a small body and horned male (when fed under sub-optimal nutritional condition) or a large body and horned male (when fed under optimal nutritional condition). The inserts represent the frequency distribution of body size and head horn size. Image derived from: Moczek et al. (14)

taurus are a secondary sexual trait that develops only in males (sexual dimorphism), and the development is also dimorphic within males (polyphenism) (12). Here, nutritional conditions during larval periods affect the size of horns: beetles develop large horns when exposed to optimal nutritional conditions while small horns or even no horns under sub-optimal nutritional conditions (13) (Figure 1). Intermediate phenotypes are relatively rare in natural populations of *O. taurus* due to large and small males utilizing specific mating tactics. Typically, large males easily compete with small males whereas small males take on a less aggressive, sneaking behavior to gain access to females (Figure 2) (15). In this case, evolution favors extreme phenotypes therefore males with intermediate phenotypes are scarce than the other two morphs (14).

O. taurus, like other beetle species, undergo complete metamorphosis with an egg, larval, pupal, and adult stages. The egg laid in the brood balls hatch in about 2-3 days, and beetles pass through three instars. The three instars represent the feeding periods of beetles, where beetles feed on the dung provided by parents. Then, in about 3-4 weeks larva sheds its rigid exoskeleton and molts into a pupa. Mainly, for chapter 1, we focused on the prepupa stage between the third instar and pupa stage. The specific time point is about 48 hours after beetles enter the prepupal stage (referred to as PP2 hereafter). At this stage, beetles have finished feeding, and the horns develop by cells undergoing rapid division and growth; therefore, their horn sizes have already been determined at this stage. Thus, the body mass during PP2 can be used to predict future adult body and horn size. Finally, in about 10-12 days, the pupa develops into adult beetles (Figure 3).

Figure 2: Image showing beetle behavior. Beetles dig tunnels in the tightly packed soil near the dung heap. The guarding males (large horned males) guard the tunnels and involve in rigorous fights with their rivals to gain possession over female mating partners. Sneakers (small horned males) adopt an alternate sneaking behavior to reach the females. The females roll dung balls, famously known as brood balls, and lay eggs in them. Image derived from Emlen et al. (15)

Figure 3: *O. taurus* life cycle showing larva, prepupa2, pupa, and adult stage.

At the pupal stage, the horn development is mostly finished, and the horn size is very similar to that of adults. However, later in pupal stage, horns are remodeled slightly to form a pair of strongly curved and more slender adult horns (Compare pupa and adult images in Figure 3). The pupal thoracic horn is obliterated by programmed cell death and is not present in adults as it is likely to be involved in the shedding of the larval head capsule (16,17).

Previous studies have shown that horns and legs are similar in the gene expression patterns (leg-horn relationship) (18). For instance, limb patterning genes such as *Distal-less* and *homothorax* are also involved in horn development (19). At the same time, in *Drosophila*, the male external genitalia share some essential genes such as *wingless* and *decapentaplegic* with legs during the onset of development, suggesting it shares the evolutionary origin with legs as well (genitalia-leg relationship) (20). Furthermore, the sex determination gene *doublesex* is responsible for the development of genitalia and horns in *O. taurus* (genitalia-horn relationship) (21). Thus, to fully understand the origin of horns, a transcriptomic analysis is critical to understand the genetic underpinnings of both genitalia and horn development.

The transcriptomic analysis also provided us with a list of appendage patterning genes and their expression patterns. We detected some wingless signaling genes such as *Wnt-1*, *frizzled-2*, *frizzled-4*, and *low-density receptor-related protein 6* (LRP6, also known as *arrow*). Not much is known about the role of Wingless signaling pathway in *O. taurus* except a study on the role of a transcription factor, *pangolin* (*pan*) (22). Thereby, to further explore the potential role of this pathway in beetle horn development, we investigated the function of gene *arrow* by RNA interference-mediated gene knockdown. This gene is present right at the beginning of the pathway along with eight paralogs of Frizzled (Fz) receptors. Since investigating all eight Fz paralogs is not feasible, therefore, for now we analyzed the developmental roles of *arrow* in *O. taurus*. A brief explanation about the research is provided in figure 4.

Figure 4: Summary flowchart of the two approaches used in this study. The first approach involves a transcriptome-wide analysis using different tissues, stages, and conditions to analyze its gene expression pattern to comprehend horn development. The second approach involves a targeted gene approach to study its role in beetle horn development.

Chapter 1: Transcriptome-wide analysis of the gene expression patterns across tissues in the dung beetle, *Onthophagus taurus*

Background:

Onthophagus taurus and its horns have been used to address the genetic underpinnings of novel trait development in the Kijimoto lab due to its availability, relatively short life cycle, and availability of its partial genomic as well as some transcriptomic data. As mentioned earlier, past research showed that legs are closest to horns in terms of gene expression patterns in *O. taurus*. It has been established that the sex determination gene *doublesex* is responsible for the development of genitalia and horns (genitalia-horn

relationship) (21). Research also indicates ablation of genital primordia during larval period resulted in larger horns than the beetles would have developed (23). The ablation of the genital disc could be responsible for the potential trade-off of resources available to the genitalia and thereby possibly share the developmental processes with horns which are at the opposite end of the animal. This further leads to a notion of genitalia being a candidate for the origin of horns. To test this hypothesis, we performed a transcriptomic analysis utilizing different tissues across stages and nutritional conditions.

The tissues utilized for RNAseq are polyphenic head horns (nutritional conditions drastically affect its size), non-polyphenic thoracic horns (nutritional conditions linearly and positively correlate with its size), the external genitalia (a sexual trait but nutritional conditions do not affect its size), and non-horn appendage, legs in the two beetle life stages, PP2, and pupa day 1 (referred to as P1 from here). Through the comparative transcriptome analysis across tissues, stages, and nutritional conditions, we aimed to understand details in the evolutionary origin of a novel trait, the beetle horns of *O. taurus*.

Hypothesis:

We hypothesize to observe gene expression similarities between horns and genitalia more than any other tissue within four groups (stage and nutritional condition) of *O. taurus*: large and small male pupa and large and small male prepupa. Our hypothesis is based on previous research (mentioned in background) where ablation of genitalia resulted in larger horns.

Materials and Methods:

Beetle Rearing:

Beetles were reared on cow dung and kept at 26°C in a regulated cycle of 16 hours of light and 8 hours of dark. Larvae were collected and transferred from brood balls to 12 well plates and kept in an incubator at 26°C. Body mass was measured at the PP2 stage, and beetles were dissected at PP2 and P1.

Body mass above 125mg was considered a large male, and anything below 120mg was considered a small male (Table 1). The mean of the two groups being statistically different with a p-value<0.05 (Table 2).

Table 1: Body mass of *O. taurus* used for this study in milligrams.

Stages	Body mass in milligrams of large males	Body mass in milligrams of small males
P1	126.6	83.4
	143.2	85.0
	146.2	103.3
	126.8	84.3
	122.7	104.8
	148.2	77.6
	145.3	83.4
	144.7	79.0
	126.3	100.9
PP2	125.5	107.3
	125.7	106.3
	136.5	105.8
	125.6	105.0
	139.2	100.5
	129.1	108.1
	125.6	97.6
	126.2	93.2
	168.6	86.5

Table 2: A two-sample t-test assuming equal variances showing a significant difference between the two group means of *O. taurus* with a p-value < 0.05. p-value=4.76E-12*

t-Test: Two-Sample Assuming Equal Variances

	Large males	Small males
Mean	135.111111	95.111111
Variance	0.149778	0.118919

Observations	18	18
Pooled Variance	0.134348	
Hypothesized Mean Difference	0	
df	34	
t Stat	10.35298236	
P(T<=t) one-tail	2.38E-12	
t Critical one-tail	1.690924255	
P(T<=t) two-tail	4.76E-12*	
t Critical two-tail	2.032244509	

Sampling:

We collected samples when beetles were in two stages: PP2 (stage right before pupation) and P1 (within 24 hours after pupation). PP2 was utilized as horn development and size determination are completed by the end of this stage. In addition, large and small males are easily distinguishable (by their look in P1 or body mass in PP2) during these stages. Four tissues were dissected from beetles for this study; sexually dimorphic head horns, which respond to nutritional input and show polyphenism. In addition, slightly sexually dimorphic thoracic horn whose size changes along with body size (continuous, linear relationship), legs with a linear relationship with body size, and sexually dimorphic genitalia that does not respond to nutritional input (24). The summarized information about tissues used in this research in Table 3.

Table 3: Tissues used for the transcriptomic analysis and the presence or absence of polyphenism and sexual dimorphism

TISSUE OF INTEREST	POLYPHENISM	SEXUAL DIMORPHISM
Head horns	✓	✓
Thoracic horns	x	✓
Legs	x	x
Genitalia	x	✓

Figure 5: A summary of all the tissues, stages, and conditions used for this study (pupal images shown)

Beetles were reared under optimal or sub-optimal nutritional conditions to induce large or small males, respectively. The same type of tissue from three beetles was pooled to increase mRNA and stored as one biological replicate. We prepared three biological replicates using nine beetles in total. The total number of samples subjected to the transcriptome analysis was 48 [3 biological replicates (each set has three beetles pooled as one) x 2 stages x 4 tissues x 2 conditions] (Figure 5).

Tissue Collection:

Beetles were dissected at the prepupa2 and pupa day 1 stage in 1X PBS buffer. The tissues of interest were dissected using surgical instruments and stored in buffer RLT plus mixture (QIAGEN, a mixture made using buffer RLT plus and β -mercaptoethanol) in 1.5 ml tubes and flash-frozen in liquid nitrogen. The extracted tissues were then stored at -80°C until further needed for RNA extraction.

RNA extraction:

Tissues were collected and RNA extracted using Qiagen RNA extraction kit and protocol. The extracted RNA was stored at -80°C until further use.

RNA-sequencing:

We used RNAseq across four different tissues (head horn, thoracic horn, legs, and genitalia) during two developmental stages (pupa and prepupa2) and two nutritional conditions (large male and small male). All RNA samples were quantified via Qubit fluorometer and run on an Agilent Bioanalyzer with an RNA Nanochip to ensure quality. Libraries were built from the high-quality RNA samples using the KAPA mRNA-Hyper plus kit from Roche Biosciences as per the manufacturer's protocol. The quantified, size determined libraries were sent to the Genomics Core Facility at Marshall University, where they were pooled in equal molar ratio and sequenced on their HiSeq 2500. All data received is used for further analysis.

Bioinformatics protocol:

Preprocessing involved an initial check of the raw reads and reads containing low-quality ends were identified and trimmed using bbduk 35.59. After that, a second quality check was performed to ensure high-quality reads for the mapping step. Preprocessed reads were mapped to the (partially annotated) reference genome using the NGS mapper STAR v2.5.2b (25). STAR a so-called split-read mapper, which allows the mapping of reads overlapping with exon-exon junctions, increased the accuracy of the mapping process. The alignment results of mapped reads were subsequently merged and used to generate mapping statistics files. The alignments were overlapped with gene models using feature Counts v1.5.3 (26). Read counts were normalized by the absolute number of mapped reads (RPM). Differential expression analysis was performed across all pair-wise combinations of conditions using the Bioconductor package edgeR v3.14.0 (27). Features with a false discovery rate (FDR) less than 0.05 were significantly differentially expressed.

Results:

We conducted a high-throughput sequencing of mRNA (RNA-seq) to quantify and compare gene expression levels across different tissues, stages, and conditions in *O. taurus* (Table 4). A stage-wise comparison was performed to observe the fluctuation in the gene expression patterns between PP2 and P1. In

parallel, a nutritional condition-wise transcriptomic analysis was performed

between large and small horned males to understand the gene expression differences/similarities in the two polyphenic morphs. The tissue specific comparison sheds more light to our main question in understanding the origin of the novel complex trait, the beetle horns.

Overall comparison across samples:

We first tried to grasp the overall gene expression pattern between the two developmental stages and conditions. Correlation heatmaps showed that the

Figure 6: An overall comparison across all samples. Spearman's correlation showing clustering of closely related samples together. The conditions (large and small samples) clustered together, whereas stages (prepupa2 and pupa) were separated. This implies gene expression similarities across conditions and differences between stages. Values range from -1 to +1.

Figure 7: Multi-dimensional scaling showing the closely related samples closer to each other distance-wise. Orange and yellow color represent pupa stage whereas dark and light blue show prepupa stage. Triangles represent head horns, squares show thoracic horn, diamonds represent legs and circles represent genitalia. Larger and small shapes represent large and small beetle samples.

conditions (large and small samples) clustered together, whereas the stages (prepupa and pupa) were less correlated (Figure 6). The multi-dimensional scaling (MDS) analysis also showed a clear separation between the two stages and no separation between large and small beetles, implying the overall variability in gene expression is explained mostly by the stages and less so with conditions (Figure 7). The numbers of differentially expressed (D.E) genes and the clustering pattern of closely related tissues between stages and conditions will be mentioned in the following sections.

Large vs. Small (nutritional conditions) comparison:

The overall sample analyses showed less variability between conditions than stages (Figures 6 and 7), thus we further investigated the D.E. genes between the two polyphenic morphs. We compared large and small pupa and detected 711 D.E. genes in the head horn. However, a much smaller number of genes were detected from the same pairwise comparison in other tissues. Specifically, 117, 69, and 92 genes were detected in thoracic horn, legs, and genitalia, respectively. In contrast, we detected only 20 genes across tissues when large and small PP2 were compared (Table 4). Out of those 20 genes, 16 genes were observed in head horns, one uncharacterized gene in thoracic horn and three genes in legs.

Table 4: Tabular representation of differentially expressed genes across large vs. small in pupa and prepupa males. False discovery rate (FDR value) ≤ 0.05

Comparison	Number of D.E. genes
Large pupa head vs. small pupa head horn	711
Large pupa thoracic vs. small pupa thoracic horn	117
Large pupa legs vs. small pupa legs	69
Large pupa genitalia vs. small pupa genitalia	92
Large prepupa head vs. small prepupa head horn	16
Large prepupa thoracic vs. small prepupa thoracic horn	1
Large prepupa legs vs. small prepupa legs	3
Large prepupa genitalia vs. small prepupa genitalia	0

Pupa vs. Prepupa comparison:

Clear separation in the MDS analyses suggested that the two stages (prepupa and pupa) be less correlated (Figure 7). With an overall variability between the two stages, we detected 2893 differentially expressed genes between large pupa (LP) and large prepupa2 (LPP) in the head horn. 2666, 3040, and 1348 D. E. genes between LP and LPP in thoracic horn, legs and genitalia, respectively. The high numbers of D.E. genes suggest the high variability between the two stages. When small pupa (SP) and prepupa2 (SPP) were compared, again relatively large numbers of D.E. genes were observed, in contrast to conditions. With a total of 2237, 2564, 2683 and 2650 D.E. genes between SP and SPP head horn, thoracic horn, legs and genitalia respectively (Table 5).

Table 5: Tabular representation of differentially expressed genes across pupa vs. prepupa2 in large and small males. False discovery rate (FDR value) ≤ 0.05

Comparison	Number of D.E. genes
Large pupa head vs. large prepupa head horn	2893
Large pupa thoracic vs. large prepupa thoracic horn	2666
Large pupa legs vs. large prepupa legs	3040
Large pupa genitalia vs. large prepupa genitalia	1348
Small pupa head vs. small prepupa head horn	2237
Small pupa thoracic vs. small prepupa thoracic horn	2564
Small pupa legs vs. small prepupa legs	2683
Small pupa genitalia vs. small prepupa genitalia	2650

Comparison between tissues:

A tissue-specific analysis was performed to understand the correlation between tissues. This analysis enumerates the number of genes shared between the four different tissues used for this study. As mentioned earlier, we also aimed to understand the gene expression

similarity or differences between the head horn and other three tissues,

namely the thoracic horn, legs, and particularly genitalia. Initially, we expected gene expression to be more similar between head horns and genitalia than legs (reasons mentioned in the introduction), however, we observed vast differences between horns and genitalia with 3313 D.E. genes and only 1046 genes between horns and legs in large vs. small pupa (Table 6). We observed more D.E. genes across different tissue comparison. For instance, large pupa head horn vs. large pupa thoracic horn, legs or genitalia showed 373, 876 and 1084 D.E. genes, respectively. Between small pupa head vs. small pupa thoracic horn, legs or genitalia, the number of D.E. genes were 1023, 557 and 2074, respectively. The data also indicated a strong tendency of tissues to be closely grouped in pupal samples, however the trend was not observed in prepupa (Figure 8). In conclusion, Spearman's correlation indicated the origin of horns independent of genitalia in gene expression patterns (Figure 8).

Figure 8: Spearman's correlation showing clustering of closely related tissues (figure shows samples from large pupa and prepupa2). Tissues like horns and legs, with less gene expression differences were clustered together as compared to genitalia in pupa. Values range from -1 to +1.

Table 6: Tabular representation total comparisons performed along with the number of D.E. genes to understand the genetic underpinnings of novel trait development in *O. taurus*. False discovery rate (FDR value) ≤ 0.05

Stage compared	Comparison	No. of D.E. genes
Pupa vs. prepupa2	Large pupa head vs. large prepupa thoracic horn	3235
	Large pupa head horn vs. large prepupa legs	3895
	Large pupa head horn vs. large prepupa genitalia	2634
	Large pupa head vs. large prepupa head horn	2893
	Large pupa thoracic vs. large prepupa thoracic horn	2666
	Large pupa legs vs. large prepupa legs	3040
	Large pupa genitalia vs. large prepupa genitalia	1348
	Small pupa head vs. small prepupa head horn	2237
	Small pupa thoracic vs. small prepupa thoracic horn	2564
	Small pupa legs vs. small prepupa legs	2683
	Small pupa genitalia vs. small prepupa genitalia	2650
	Large pupa head vs. small prepupa thoracic horn	3239
	Large pupa head horn vs. small prepupa legs	3176
	Large pupa head horn vs. small prepupa genitalia	3011
Pupa vs. pupa	Large pupa head vs. large pupa thoracic horn	373
	Large pupa head horn vs. large pupa legs	876
	Large pupa head horn vs. large pupa genitalia	1084
	Small pupa head vs. small pupa thoracic horn	1023
	Small pupa head horn vs. small pupa legs	557
	Small pupa head horn vs. small pupa genitalia	2074
	Large pupa head vs. small pupa head horn	711
	Large pupa thoracic vs. small pupa thoracic horn	117
	Large pupa legs vs. small pupa legs	69
	Large pupa genitalia vs. small pupa genitalia	92
	Large pupa head vs. small pupa thoracic horn	1219
	Large pupa head horn vs. small pupa legs	1046
	Large pupa head horn vs. small pupa genitalia	3313
	Large prepupa head vs. large prepupa thoracic horn	176

	Large prepupa head horn vs. large prepupa legs	203
Prepupa2 vs. prepupa2	Large prepupa head horn vs. large prepupa genitalia	379
	Small prepupa head vs. small prepupa thoracic horn	256
	Small prepupa head horn vs. small prepupa legs	198
	Small prepupa head horn vs. small prepupa genitalia	468
	Large prepupa head vs. small prepupa head horn	16
	Large prepupa thoracic vs. small prepupa thoracic horn	1
	Large prepupa legs vs. small prepupa legs	3
	Large prepupa genitalia vs. small prepupa genitalia	0

Discussion:

We performed comparative transcriptomic analysis to obtain knowledge for the complex evolution of novel traits, horns of *O. taurus*. The pupa and prepupa stages, large and small males (optimal and sub-optimal nutritional conditions respectively), and four different tissues: head horns, thoracic horn, legs, and genitalia were utilized in this study. We aimed to investigate similarities/differences in gene expression patterns across tissues, mainly focusing on horns and genitalia.

The clustering and MDS analyses showed clear separation in correlation and distance between stages rather than the conditions (Figures 6 and 7). Our results also showed more D.E. genes between the stages than conditions (Table 6), suggesting that many genes change their expression pattern when beetles undergo metamorphosis. In contrast, relatively fewer differences exist between the two polyphenic morphs.

Between morphs, we detected more D.E. genes in LP vs. SP (D.E. genes in head horns, thoracic horn, legs, and genitalia were 711, 117, 69, and 92 respectively) in contrast to LPP vs. SPP across tissue comparison with only 20 genes differentially expressed (D.E. genes in head horns, thoracic horn, legs, and genitalia were 16, 1, 3, and 0 respectively). Among the 711 genes differentially expressed between large

and small pupa head horn comparison, we observed a low-density lipoprotein receptor-related protein (LRP, *arrow* in *Drosophila*) expression more in small than large pupa head horns (with a log fold change of 2.97). We speculate its role in horn inhibition due to its overexpression in small beetles. The role of this gene is already established in wing and leg development of fruit flies and red flour beetles respectively (32, 39). However not much is understood about its horn developmental role in *O. taurus* which will be discussed in detail in chapter 2. Of the 16 genes that were detected in large vs. small prepupa head horns, we identified 11 genes being expressed more in head horns of large beetles. These 11 genes included three unidentified genes, two glutamate receptor genes which are known to be involved in neuronal activities within the nervous system in *Drosophila* (28). We also identified golgin subfamily A member 6-like protein 22 to be expressed about thirty times more in head horns of large males. Golgins have a role in protein transportation either in or outside the cell (29). The overexpression of these genes in large males (more than in small prepupa) might suggest the increased protein transportation in large prepupa head horns. Another gene called *Tret1* particularly sought our attention due to its known function as well as increased expression in large prepupa head horns by eight times than small prepupal head horns. *Tret1* has a role in regulating trehalose levels in the hemolymph of insects (30). The increased expression of this gene (*Tret1*) in head horn might be associating with potential breakdown of trehalose into sugars to maintain the energy metabolism required in the horn primordia of large prepupa beetles. Five genes were expressed more in small prepupa head horns than large and included one unidentified gene, three cuticle related genes and a splicing factor subunit gene.

We observed more differentially expressed genes in the pupa stage than prepupa. A possible explanation to this might be the dramatic developmental event of metamorphosis, during the pupa stage leading the same cells to express more genes in this stage. This transition from prepupa to the pupa stage therefore might be causing the gene expression differences between the two stages.

We next tested our hypothesis of the origin of horns in *O. taurus*. We expected the clustering of horns and genitalia together; however, our analyses did not indicate clear correlation between them. Instead, we observed the clustering of similar tissues such as pupal head horns, thoracic horn, and legs together (Figure 8). The number of D.E. genes when large pupa head horn was compared with large pupa thoracic horn, legs and genitalia was 373, 876 and 1084 respectively. We observed more differences in the number of D.E. genes when small pupa head horns and the other three tissues (thoracic horn, legs and genitalia) were compared with 1023, 557 and 2074 (respectively) genes. The difference in the number of D.E. genes between large and small head horns compared with thoracic horn led us to speculate that there might be an association between the two tissues. This difference might have occurred as more genes were expressed in head horns of large than small pupa. It also suggested the difference in degrees of similarity across tissues between large and small beetles. In other words, head and thoracic horns of large pupa were more similar in gene expression than small pupa. A similar pattern was observed in large and small prepupa. When comparing head horns to legs we observed less D.E. genes than genitalia in both stages and conditions. This suggests the three tissues, head horns, thoracic horn and legs are closer in gene expression. Thereby, we conclude that head horns and genitalia differ in gene expression more than legs in the pupal stage of these morphs.

To summarize, my research filled some gaps in understanding the basis of horns by highlighting the differences in gene expression between horns and genitalia, which we initially hypothesized to be similar. Further investigation is still required to conclude the close relatedness of legs to horns.

Future plans:

Our future work will include a Gene ontology enrichment analysis or GO-term analysis to look for functions of genes enriched between stages, conditions, and tissues. Moreover, since this study is novel and not much research has been done on the PP2 stage of this beetle, other tissues such as wings,

abdomen, etc., can be used further to understand the evolution of these horns. We will also consider knocking down some genes, such as *Tret1*, and others from our list to further examine their beetle development roles.

Chapter 2: Targeted gene approach: "arrow"

Background:

The comparative transcriptome analyses provided the list of genes involved in beetle development. Among many candidates, we focused on the genes involved in Wingless signaling pathway such as Wnt-1, *frizzled-2*, *frizzled-4*, low-density lipoprotein receptor-related protein 6 (LRP6) (also known as *arrow* in *Drosophila*). Signaling pathways often operate in conjunction with other pathways (31). During *Drosophila* embryogenesis, *wg* affects the expression of another signaling protein, Hedgehog (Hh); *wg* is responsible for *hh*'s continued expression (32). Previously, the Hedgehog signaling pathway was shown to be responsible for horn suppression in *Onthophagus taurus* (33). Thus, we aimed to investigate the role of a Wingless signaling pathway gene called *arr* in the horn development. Detailed information about Wg pathway as part of the rationale for studying *arrow* is provided below.

The Wingless/Wnt signaling pathway is an evolutionary conserved genetic pathway in insects. The proteins involved in this pathway have various developmental and cell biological processes such as cell proliferation, differentiation, and polarity

Figure 9: Visualization of the Wingless pathway. The figure on the right shows the binding of Wingless signaling molecule with Frizzled receptors (Fz receptors are comprised of eight transmembrane receptors). The Fz-Wg complex now associates with Arrow, thereby causing signal transduction from outside the cell to the cytoplasm and consequently targeting gene transcription in the nucleus. The left figure shows the signal not transducing in the cell without the binding of Fz, Wg and Arr.

(32). The morphogen protein Wingless (Wg) is secreted from cells into extracellular space and received by cells that express receptor proteins. It binds to two transmembrane receptors, the Frizzled (Fz) which

includes eight transmembrane receptors and the LRP (Low-density lipoprotein receptor-related protein) family. The Fz-Wg complex associates with Arrow (Arr) (the *Drosophila* homolog of LRP5/6), leading to transducing signals to the cytoplasm, consequently regulating gene transcription in the nucleus (34) (Figure 9).

The endocytic degradation of morphogen proteins is required to create a signaling gradient on the surface of epithelial cells during development (35). Wg degradation was previously believed to be mediated by the cooperation of Arr and Fz receptors, however researchers later concluded that Arr was solely responsible for Wg degradation in *Drosophila* wing imaginal discs (36).

Previous studies of *arr* knockdown in *Drosophila* resulted in Wg accumulation in the cells (37) which has been shown to inhibit cell proliferation in the wing imaginal disc (38). In the red flour beetle, *Tribolium castaneum*, the role of Wingless receptor Arr is known in axis elongation and leg development (39). The function of these genes is studied primarily during embryogenesis; however, the role of *arr* in *O. taurus* during the late development (i.e., after embryogenesis) or in the context of horn development is not known.

In the transcriptomic analyses, along with *hedgehog* we also observed the expression of *arrow* more in small head horns than large beetles suggesting their roles in horn inhibition which will be explored later in the chapter. We also detected the Wg receptors, *frizzled-2* and *frizzled-4*, but due to the presence of paralogs with similar gene sequence, it is not feasible to study the function of these genes during the limited research time. Therefore, this research includes knocking down the gene *arrow* that encodes the low-density lipoprotein receptor-related protein (LRP) to identify its role and the role of Wg signaling in beetle development (40). We also studied the potential dosage effect of *arr* knockdowns in the horn size determination by utilizing 1µg, 2µg, and 5µg RNAi knockdowns.

Hypothesis:

By knocking down *arrow* we will observe horn inhibition in *O. taurus*. The rationale includes Wingless protein accumulation in the cells and thereby prolonged/constitutional expression of *hedgehog* (as mentioned in the background) that suppresses the horn growth.

We should observe similarities in results as *T. castaneum*, where the legs of these beetles were affected upon *arrow* knockdown. We also expect to observe some wing deformations as this gene is involved in the Wingless signaling pathway responsible for the development of wing imaginal disc in *Drosophila* (32).

Materials and Methods:

Animal husbandry

Third instar was utilized to inject dsRNA. After injecting, the beetles were weighed at PP2 to predict future body sizes. Images were captured at the pupal stage for size measurement, then stored in 70% EtOH for preservation after becoming an adult.

Gene isolation

The *arr* sequence was obtained from the partially annotated *O. taurus* genome database (41). To confirm the sequence, RT-PCR was utilized to amplify *arr* cDNA fragments from male RNA samples. The primers were designed using the gene-specific region (Figure 11) with Oligo Analyzer Tool. The primer sequence used was- Otarr-speF1(forward primer): 5'- ATACAGGCATTATAGGGCAAT -3' and OtarrR1(reverse primer): 5'- ACAACTTTCGCTTTGACAAT -3'. First, the gene-specific fragment was amplified using PCR cycle at 98°C (denaturing temperature) for 30 sec, 57°C (annealing temperature) for 30 sec and 72°C (extension temperature) for 30 sec with 30 cycles. The amplified fragment (Figure 11) was then cloned into a pSC-A vector (Figure 10) using the StrataClone PCR Cloning Kit (Agilent). Followed by

PCR confirmation, the plasmid was purified using the GeneJET MiniPrep Kit (Fisher). Finally, the purified

Figure 10: pSC-A-amp/kan PCR cloning vector along with PCR product insertion site region, T3, T7 promoters, M13 forward and reverse primer binding sites.

plasmid was confirmed as *arrow* after sequencing (Eurofins Genomics).

The isolated gene-specific region was amplified from the plasmid vector with M13 forward and reverse primers. The PCR product contained the T3 and T7 promoters on 5'- and 3'- end, respectively, and was used as a template for in vitro transcription (IVT). T3 and T7 RNA polymerases were used to generate two single-stranded RNA fragments complementary to each other (MegaScript RNAi Kit, Thermo Fisher Scientific). The single-stranded RNA fragments generated by T3 or T7 RNA polymerases were mixed in equal mass, denatured at 75°C for 10 mins, and then chilled on ice immediately. The mixture was then incubated in a water bath at 80°C overnight until room temperature was attained. The annealed double-stranded RNA (dsRNA) concentration was measured and confirmed by gel electrophoresis. The dsRNA

Figure 11: The gene model of *arrow*. The dark blue boxes represent the exons. Introns are depicted in light blue bars. The expected length of the gene is around 4000 base pairs. The gene specific region used for dsRNAi is 163 bp long. The black arrows represent the primer set used for gene amplification.

was stored at -80°C until injection.

Beetle larvae were injected with three different amounts, 1µg, 2µg, and 5µg in 3µl of injection buffer. The injection buffer comprised of 1mM sodium phosphate buffer (which included 510µl of 0.2M NaH₂PO₄ and 490µl of 0.2M Na₂HPO₄ to achieve a pH of 6.8) to 5mM KCl. After the injections, larvae were

fed by cow manure, and the body mass was measured when they reached the prepupa2 stage. Beetles were later collected once they emerged as adults and preserved in 70% EtOH.

For the control injections, beetles were reared in the same way as *arr* treated beetles. The control beetles were injected with dsRNA generated from a 261bp PCR product derived from a pSC-A-amp/kan vector without any insertions (Figure 10). Other reactions to generate control dsRNA were performed similarly to *arr* dsRNA. A total of 1 μ g/ 3 μ l dsRNA was injected into control larvae during the third instar. Controls were similar to wild type beetles in phenotype. We injected beetles with control dsRNA to single out any phenotypic defects caused by injections.

Beetle imaging and measurement

The images were captured using the Scion camera attached to Leica microscope of both pupa and adult beetles. Beetles were measured using the ImageJ software (42). As reported in Beetle larvae were injected with three different amounts, 1 μ g, 2 μ g, and 5 μ g in 3 μ l of injection buffer. previous research, horns were measured from the end of the eye to the tip of the horn (43). Thoracic width (widest part of the thorax) was utilized to represent the body size in pupa and adult stages (44) (Figure 12). These measurements were taken across all injected beetles.

Figure 12: Beetle measurements in adult and pupa stages A) Adult stage. Top: Image showing the measurement of body size. Bottom: Image showing measurement of head horn size. B) Pupa stage. Top left: Measurement of head horn size. Top right: Measurement of body size. Bottom: Measurement of thoracic horn size, which develops only in the pupa.

Statistical analyses

We compared horn and body size between control (for pupa: $n = 70$; for adults $n = 80$) and *arr* treated (for pupa: $n = 61$; for adults: $n = 56$) for both pupa and adult stages. The *arr* treated beetles were further divided by the amount of dsRNA knockdowns. On this basis, we had $1\mu\text{g}$, $2\mu\text{g}$, and $5\mu\text{g}$ injections for *arr* groups (as mentioned above). We also analyzed the difference in phenotypes between control and *arr* treated beetles. JMP was utilized to show the difference in mean horn size, determine the distribution of each group, and mimic orthogonal contrasts.

The continuous variables (horn and body size) were examined for normal distribution of residuals, using Shapiro-Wilk

Figure 13: Head horn size distribution in control and *arr* treated pupa. p-value: $<.0001^*$ and $.061$ respectively.

W test, both in pupa and adult males. As expected, we found

small p-values indicating that horn size data were not normally distributed in most of the groups (control, treated with *arr* $1\mu\text{g}$, $2\mu\text{g}$ and $5\mu\text{g}$), (Figure 13).

Due to the lack of normality, a one-way nonparametric analysis (Kruskal Wallis) on all four groups (control, *arr* $1\mu\text{g}$, $2\mu\text{g}$ and $5\mu\text{g}$) was performed to compare horn and body size between control (for pupa: $n = 70$; for adults $n = 80$) and *arr* treated (for pupa: $n = 61$; for adults: $n = 56$) beetles in

Table 7: Kruskal-Wallis tests to show difference in score means of the adult horn and body size between different treatment groups.

Injection	Sample size	Score mean for head horns	Score mean for body size
Control ($1\mu\text{g}$)	80	77.1250	77.1813
<i>arr</i> $1\mu\text{g}$	9	21.6667	17.3333
<i>arr</i> $2\mu\text{g}$	28	55.6607	57.9107
<i>arr</i> $5\mu\text{g}$	19	73.2895	71.7895

pupa and adult stages. Kruskal-Wallis test was performed to measure if there was any inequality among them (Table 7). To further validate the inequality among treatments we performed Chi-square test which confirmed the difference through insignificant value ($p < 0.05$).

After the initial analysis where all *arr* groups combined were compared to control, two additional contrasts similar to the orthogonal analysis in ANOVA were performed. We utilized a nonparametric test in an orthogonal fashion to examine if there is any effect among the different doses of *arr* knockdowns in horn size of beetles. Specifically, the additional contrasts were: *arr* 1 μ g vs. *arr* 2 and 5 μ g, then *arr* 2 μ g vs. 5 μ g (Table 8).

Table 8: Tabular representation of orthogonal set used for this setup. 1,2, and 5 represent 1 μ g, 2 μ g, and 5 μ g injections.

		Control	<i>arrow</i> 1	<i>arrow</i> 2	<i>arrow</i> 5	Row summation
Contrast1	Control vs All <i>arrow</i> (1, 2, 5)	3	-1	-1	-1	0
Contrast2	<i>arrow</i> 1 vs (<i>arrow</i> 2 and 5)	0	2	-1	-1	0
	check if Contrast 1 and 2 are orthogonal, cross products	0	-2	1	1	0
Contrast3	<i>arrow</i> 2 vs <i>arrow</i> 5	0	0	1	-1	0
	check if Contrast 3 and 1 are orthogonal, cross products	0	0	-1	1	0
	check if Contrast 3 and 2 are orthogonal, cross products	0	0	-1	1	0

Use of orthogonal contrasts in the nonparametric application is referred to as nested orthogonal contrasts in analysis of rank data (45, 46). A set of orthogonal contrasts represent independent

comparisons (uncorrelated and not confounded). Mathematically, contrasts are considered orthogonal if the sum of the constants within each contrast is zero and also the summation of the cross-element products is zero (matrix multiplication of all contrast with each other) These conditions were validated by hand calculation.

Further regression analysis was performed to determine the slope differences between control and *arr* treated groups to observe the relationship between doses (control, 1, 2 and 5µg *arr* groups). We first compared the slopes for all *arr* treatments (1,2 and 5µg) with controls and determined a significant negative association with high p-value >0.05 (p-value for adult body size comparison = 0.3303; p-value for adult head horn size comparison = 0.3875). A similar trend was observed in pupa. We next compared all *arr* groups without controls as we observed inequality in the Kruskal-Wallis test and wanted to detect the possible group responsible. Linear regression line (positive slope) with a small p-value was observed across all *arr* regression (p-value for adult body size = 0.0071; p-value for adult head horn = 0.0043) suggesting all *arr* treated groups body and head horn size to increase with an increase in dosage amount.

Data were analyzed using JMP software (JMP®, Version Pro 14.0, SAS Institute Inc., Cary, NC, Copyright ©2015). The significance criterion alpha for all tests was 0.05.

Results:

The size of the gene *arrow* in *O. taurus* inferred from the partially annotated genome is 4242 nucleotides (41). The entire *O. taurus arr* sequence was then subjected to BlastX, which resulted in 44% and 97% identity with *D. melanogaster* and *T. castaneum*, respectively. In conjunction with the sequence identity we also observed similar wing and leg defects in previous *Drosophila* and *T. castaneum* embryo researches on *arr* knockdown (mentioned in other notable phenotypes) confirmed the sequence is more likely to be the *arr* ortholog in *O. taurus*.

Kruskal-Wallis tests showed horn and body measurements of 1 μ g injections with a smaller body and horn size with a score mean of 17.333 and 21.667 respectively as compared to controls (head horn and body size score means are 77.1250 and 77.1813 respectively). As mentioned earlier, we performed Chi-square tests to confirm the inequality determined from Kruskal-Wallis test was not by chance. A small p-value of 0.002 showed that among the four groups (all *arr* treatments and controls) there is an inequality. Further, to observe the group responsible for the difference we utilized orthogonal contrasts (discussed in the following sections) to understand the possible dosage effect of this gene in our beetles.

Contrast 1 – Control vs all *arr* groups:

The first comparison was carried out between control and *arr* RNAi (1 μ g, 2 μ g, and 5 μ g injection). We observed that the average body and horn sizes of 1 μ g *arr* knockdowns were significantly smaller than controls (Figure 14).

Figure 14: One-way analysis test for control and *arr* treated beetles in adult horn and body sizes. Light green circles, light blue inverted triangles and dark blue diamonds represent 1 μ g, 2 μ g and 5 μ g injected beetles.

Contrast 2: *arr* 1 μ g vs 2 μ g and 5 μ g:

The overall horn and body average was smaller for *arr* treated beetles than controls (Figure 15). Contrast 2 tested if the different amount of *arr* dsRNA injection (1 μ g to 2 μ g and 5 μ g) affected the body and horn size. This contrast was utilized as the mean value for 1 μ g injections was much smaller than the other two. As expected, one-way analysis showed smaller body and horn sizes for both pupa and adults in 1 μ g injections (Figure 15).

Figure 15: One-way test results showing the averages for 1 μ g vs. 2 μ g and 5 μ g *arr* treated beetles in adult horn and body sizes. P-values: 0.0017*(head horn) and 0.0005*(body size)

Contrast 3: *arr* 2 μ g vs 5 μ g:

We observed an overlap between the 2 μ g and 5 μ g injections in the first contrast (Figure 15). Therefore, contrast 3 was tested to determine if the 2 μ g and 5 μ g injected beetles show significant

Figure 16: One-way test results showing the averages for 2 μ g and 5 μ g *arr* treated beetles in adult horn and body sizes.

differences in the horn and body size. One-way test results suggested average horn and body size not significantly different between the groups (Figure 16).

Regression analysis:

We also performed regression analysis to determine the effect of different doses used. First, all the groups were considered to observe the association among them. We observed a negative association for head horn and body across pupa and adult suggesting a decrease in size with increase in dosage (Figure 17). However, large p-values >0.05 (p-value for head horn size = 0.3875; p-value for body size = 0.3303) indicated no statistical significance for our results.

Figure 17: Linear regression line with a negative slope with controls. 0, 1, 2 and 5 on x-axis represents control, 1, 2 and 5 μg injected beetles.

Regression analysis without using the control groups showed a positive association suggesting an increase in head horn and body sizes with dosage increase in both pupa and adults with statistical

significance (p-value for head horns = 0.0043; p-value for body size = 0.0071) (Figure18).

Figure 18: Linear regression line with a positive slope without controls. 0, 1, 2 and 5 on x-axis represents control, 1, 2 and 5 μ g injected beetles.

Other notable phenotypes:

arr treated beetles showed other notable phenotypes other than the horn and body size differences. The treated beetles showed some severe to mild wing defects (Figure 19). We considered an elevated elytra (compare with controls in figure 19) and more than two holes on the elytra as severe damages whereas mildly damaged wings had an elevated elytra and two or less holes. Out of the total 56 adults, we observed 19 with severe (33%), 22 with mild (39%) and 16 males with no (28%) wing defects, which included severely damaged and shortened elytra. We also observed leg defects in the males, which included twisted and narrowed tibia and a twisted tarsus (Figure 19).

A

B

Figure 19: Wing and leg defects. A) Wing defects: Control (left) and *arr* treated with mild (center) and severe (right) defects. Arrow heads show the elevated elytra and holes on the elytra in *arr* treated beetles. B) Leg defects: Control(left) and *arr* treated(right). Twisted tarsus and narrow tibia highlighted with red arrow heads.

Discussion:

Our transcriptomic data provided us with a list of genes expressed across tissues. Among all detected differentially expressed genes, we observed some Wingless signaling genes and further investigated a specific gene called *arr*. The Wingless pathway starts by the binding of Wg proteins to its Frizzled and Arrow receptors, which further leads to signal transduction to the cytoplasm and thereby causing gene transcription in the nucleus. Arrow is responsible for creating a signaling gradient by the endocytic degradation of Wg in wing imaginal discs of *Drosophila* (36). We utilized RNAi to knockdown *arr* to examine its horn developmental role in *O. taurus*. Our data suggested the possible function of *arrow* in determining the horn and body size in *O. taurus* as we observed an overall reduction in beetle horn and body size. This might have occurred due to the interaction of genetic signaling pathways such as Wingless and Hedgehog. The role of Hedgehog pathway is known in both horn development and polyphenism, and mainly causing horn suppression in small beetles. The knockdown of *arr* might have caused the accumulation of Wingless protein which would result in prolonged/constitutive expression of *hedgehog* ultimately leading to horn inhibition.

Intriguingly, knocking down *arr* by RNAi also showed a potential dosage-dependent effect in *O. taurus*. As mentioned earlier, we injected beetles with three different doses (1µg, 2µg, and 5µg) to examine its effect. Three different orthogonal contrasts were utilized to observe the effect of doses on development. We observed only smaller horns and thoracic width (body size) in 1µg injections, whereas we did not detect any significant difference between 2µg, 5µg

Figure 20: Flowchart to show dosage effect of the gene *arr*. When high levels of *arr* mRNA is present with 1µg injection we observe smaller horn and body sizes, whereas low mRNA levels with 2 and 5 µg injections had both small and large horns and body sizes

knockdowns and controls. This implies the presence of a possible threshold effect in the expression of this gene. Specifically, with weaker (1 μ g) knockdowns, when the gene expression level is higher (more *arr* mRNA), the threshold was not reached and thus, it resulted in smaller horn and body size. In contrast, with 2 μ g and 5 μ g injections threshold was reached, and resulted in both large as well as small horns and body size (Figure 20), a trend also observed in controls.

There were some limitations in our statistical analyses. Due to the constraint of degrees of freedom in orthogonal test (degree of freedom = $n-1$; $n = 4$ (control, *arr* 1, 2 and 5 μ g)) we can only perform limited contrasts and were not able to compare each *arr* group with controls. However, we observed a significant difference between the control and *arr* treated groups in the first contrast, showing an overall reduction in the average of both horn and body sizes in the *arr* treated beetles.

Regression analysis between the *arr* treated groups showed a statistically significant linear and positive relationship between 1,2 and 5 μ g treated beetles. This indicated an increase in horn and body size with increasing amounts of injection. Contrary to this we observed a linear and negative slope when all the groups were compared along with controls with a p-value more than .05 suggesting no statistical significance.

The Wingless signaling pathway plays an essential role in forming the wing imaginal disc in *Drosophila* (as mentioned in hypothesis) (32). We showed here that *arr* knockdowns in *O. taurus* is also responsible for the development of wing during the late development (larva and pupa). We observed 33% and 39% of our beetles with severe and mild defects, which included damaged elytra and hindwings. Among the 1 μ g injected beetles we observed 6 beetles with severe and 3 with mild damages. For the 2 μ g injections we observed 9 beetles with severely damaged wings and 11 with mild damages. 5 μ g injected beetles included more beetles with mild (8 beetles showing mild defects) damages than severe (4 severely defected beetles). These results suggest the possible role of *arr* in Wingless accumulation, thereby causing

the wing defects, a phenotype similar to *arr* knockdown in *Drosophila* (37). We also observed similar leg defects as *T. castaneum* (39) which included twisted tibia and tarsus. The defect in other tissues also underscores the role of gene co-option in development.

Future plans:

To strongly support the dosage dependent role of this gene we plan to inject more beetles with 1ug dsRNA to increase our sample size. We also plan on utilizing qPCR as that will quantify the role of *arr* and the possible effects it has on other genes involved in the pathway.

Concluding remarks:

This research fills in some major gaps in our understanding of the novel complex trait, the horns of *O. taurus*. Chapter 1 provided a detailed list of genes differentially expressed across four tissues and highlighted the gene expression patterns in those tissues. Our results indicated that the horn is “well-defined” in its identity in the pupal stage, and it is not clearly close to genitalia unlike we expected. At the same time, four tissues are not clearly separated in prepupa, which indicates they are “less-defined” in the identity of tissues. Our results also showed high numbers of differentially expressed genes between horns and genitalia suggesting it may not be the closest origin of horns. Further research is still required to investigate the tissue relatedness in terms of gene expression pattern. Chapter 2 highlights the horn developmental role of the gene *arr* which belongs to the Wingless signaling pathway, that was not explored in our beetles before. We also propose a potential threshold effect of this gene in the development of beetles. Further work is needed to fully understand the dosage effect of *arr* in our beetles.

REFERENCES

1. Muller, G B, and G P Wagner. "Novelty in Evolution: Restructuring the Concept." *Annual Review of Ecology and Systematics* 22, no. 1 (1991): 229–56.
<https://doi.org/10.1146/annurev.es.22.110191.001305>.
2. Darwin, C. "The Origin of Species and the Descent of Man and Selection in Relation to Sex." http://darwin-online.org.uk/converted/pdf/1889_Descent_F969.pdf.
3. "Wallace, A. R. 1895. Natural Selection and Tropical Nature: Essays on Descriptive and Theoretical Biology. London: Macmillan."
http://darwinonline.org.uk/converted/Ancillary/1895_NaturalSelection_A238.html.
4. Lameere, A. (1904). L'évolution des ornements sexuels. *Bulletins de l'Académie royale des sciences, des lettres et des beaux-arts de Belgique*, (12), 1327-1364.
5. Doane, R.W. 1913. "The Rhinoceros beetle *Oryctes rhinoceros* in Samoa." *Journal of Economic Entomology* <https://books.google.com/books?hl=en&lr=&id=wgi0AAAAMAAJ&oi=fnd&pg=PA438-IA11&dq=Doane+R+W+1913&ots=Jj0RFUvKjk&sig=jzUCLCcpLckJQMUjI5pE7TRH-Xs#v=onepage&q=Doane%20R%20W%201913&f=false>.
6. Arrow, G.H. 1951 "Horned Beetles - A Study of the Fantastic in Nature | Gilbert J. Arrow | Springer." <https://www.springer.com/gp/book/9789401757751>.
7. Eberhard, William G. "Fighting Behavior of Male *Golofa Porteri* Beetles (Scarabeidae: Dynastinae)." *Psyche: A Journal of Entomology* 84, no. 3–4 (January 1, 1977): 292–98.
<https://doi.org/10.1155/1977/19030>.
8. Pfennig, David W., Matthew A. Wund, Emilie C. Snell-Rood, Tami Cruickshank, Carl D. Schlichting, and Armin P. Moczek. "Phenotypic Plasticity's Impacts on Diversification and Speciation." *Trends in Ecology & Evolution* 25, no. 8 (2010): 459–67. <https://doi.org/10.1016/j.tree.2010.05.006>.
9. Moczek, Armin P., Sonia Sultan, Susan Foster, Cris Ledón-Rettig, Ian Dworkin, H. Fred Nijhout, Ehab Abouheif, and David W. Pfennig. "The Role of Developmental Plasticity in Evolutionary Innovation." *Proceedings of the Royal Society B: Biological Sciences* 278, no. 1719 (2011): 2705–13.
<https://doi.org/10.1098/rspb.2011.0971>.
10. Fordyce, J. A. "The Evolutionary Consequences of Ecological Interactions Mediated through Phenotypic Plasticity." *Journal of Experimental Biology* 209, no. 12 (June 15, 2006): 2377–83.
<https://doi.org/10.1242/jeb.02271>.
11. Whitman, Douglas, and Anurag Agrawal. "What Is Phenotypic Plasticity and Why Is It Important?" In *Phenotypic Plasticity of Insects*, edited by Douglas Whitman and T Ananthakrishnan. Science Publishers, 2009. <https://doi.org/10.1201/b10201-2>.
12. Moczek, Armin P. "Horn Polyphenism in the Beetle *Onthophagus Taurus*: Larval Diet Quality and Plasticity in Parental Investment Determine Adult Body Size and Male Horn Morphology." *Behavioral Ecology* 9, no. 6 (January 1, 1998): 636–41. <https://doi.org/10.1093/beheco/9.6.636>.
13. Emlen, Douglas J., John Hunt, and Leigh W. Simmons. "Evolution of Sexual Dimorphism and Male Dimorphism in the Expression of Beetle Horns: Phylogenetic Evidence for Modularity, Evolutionary Lability, and Constraint." *The American Naturalist* 166 Suppl 4 (October 2005): S42-68.
<https://doi.org/10.1086/444599>.

14. Moczek, Armin P., and Douglas J. Emlen. "Male Horn Dimorphism in the Scarab Beetle, *Onthophagus Taurus*: Do Alternative Reproductive Tactics Favour Alternative Phenotypes?" *Animal Behaviour* 59, no. 2 (February 2000): 459–66.
15. Emlen, Douglas J. "Integrating Development with Evolution: A Case Study with Beetle Horns: Results from Studies of the Mechanisms of Horn Development Shed New Light on Our Understanding of Beetle Horn Evolution." *BioScience* 50, no. 5 (May 1, 2000): 403–18. [https://doi.org/10.1641/0006-3568\(2000\)050\[0403:IDWEAC\]2.0.CO;2](https://doi.org/10.1641/0006-3568(2000)050[0403:IDWEAC]2.0.CO;2).
16. Moczek, Armin P., Tami E. Cruickshank, and Andrew Shelby. "When ontogeny reveals what phylogeny hides: gain and loss of horns during development and evolution of horned beetles." *Evolution* 60, no. 11 (2006): 2329–41. <https://doi.org/10.1111/j.0014-3820.2006.tb01868.x>.
17. Kijimoto, Teiya, Justen Andrews, and Armin P. Moczek. "Programed Cell Death Shapes the Expression of Horns within and between Species of Horned Beetles." *Evolution & Development* 12, no. 5 (2010): 449–58. <https://doi.org/10.1111/j.1525-142X.2010.00431.x>.
18. Kijimoto, Teiya, James Costello, Zuojian Tang, Armin P. Moczek, and Justen Andrews. "EST and Microarray Analysis of Horn Development in *Onthophagus* Beetles." *BMC Genomics* 10, no. 1 (October 30, 2009).
19. Moczek, Armin P., and Debra J. Rose. "Differential Recruitment of Limb Patterning Genes during Development and Diversification of Beetle Horns." *Proceedings of the National Academy of Sciences* 106, no. 22 (June 2, 2009): 8992. <https://doi.org/10.1073/pnas.0809668106>.
20. Casares F, Sánchez L, Guerrero I, Sánchez-Herrero E. The genital disc of *Drosophila melanogaster*. : I. Segmental and compartmental organization. *Dev Genes Evol.* 1997 Oct;207(4):216-228. doi: 10.1007/s004270050110. PMID: 27747420.
21. Kijimoto, Teiya, Armin P. Moczek, and Justen Andrews. "Diversification of Doublesex Function Underlies Morph-, Sex-, and Species-Specific Development of Beetle Horns." *Proceedings of the National Academy of Sciences* 109, no. 50 (December 11, 2012): 20526–31. <https://doi.org/10.1073/pnas.1118589109>.
22. Wasik, Bethany R., and Armin P. Moczek. "Pangolin Expression Influences the Development of a Morphological Novelty: Beetle Horns." *Genesis* 50, no. 5 (May 1, 2012): 404–14. <https://doi.org/10.1002/dvg.20814>
23. Moczek, Armin P., and H Frederik Nijhout. "Trade-Offs during the Development of Primary and Secondary Sexual Traits in a Horned Beetle." *The American Naturalist* 163, no. 2 (February 2004): 184–191. <https://doi.org/10.1086/381741>.
24. Parzer, Harald F., and Armin P. Moczek. "Rapid Antagonistic Coevolution Between Primary and Secondary Sexual Characters in Horned Beetles." *Evolution* 62, no. 9 (2008): 2423–28. <https://doi.org/10.1111/j.1558-5646.2008.00448.x>.
25. Dobin, Alexander, Carrie A. Davis, Felix Schlesinger, Jorg Drenkow, Chris Zaleski, Sonali Jha, Philippe Batut, Mark Chaisson, and Thomas R. Gingeras. "STAR: Ultrafast Universal RNA-Seq Aligner." *Bioinformatics* 29, no. 1 (January 1, 2013): 15–21. <https://doi.org/10.1093/bioinformatics/bts635>.
26. Liao, Yang, Gordon K. Smyth, and Wei Shi. "FeatureCounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features." *Bioinformatics* 30, no. 7 (April 1, 2014): 923–30. <https://doi.org/10.1093/bioinformatics/btt656>.
27. Robinson, Mark D., Davis J. McCarthy, and Gordon K. Smyth. "EdgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data." *Bioinformatics* 26, no. 1 (January 1, 2010): 139–40. <https://doi.org/10.1093/bioinformatics/btp616>.
28. Ultsch, A, C M Schuster, B Laube, P Schloss, B Schmitt, and H Betz. "Glutamate Receptors of *Drosophila Melanogaster*: Cloning of a Kainate-Selective Subunit Expressed in the Central Nervous System."

- Proceedings of the National Academy of Sciences of the United States of America* 89, no. 21 (November 1, 1992): 10484–88. <https://doi.org/10.1073/pnas.89.21.10484>.
29. Lowe, Martin. "The Physiological Functions of the Golgin Vesicle Tethering Proteins." *Frontiers in Cell and Developmental Biology* 7 (June 18, 2019): 94–94. <https://doi.org/10.3389/fcell.2019.00094>.
 30. Kanamori, Yasushi, Ayako Saito, Yuka Hagiwara-Komoda, Daisuke Tanaka, Kanako Mitsumasu, Shingo Kikuta, Masahiko Watanabe, Richard Cornette, Takahiro Kikawada, and Takashi Okuda. "The Trehalose Transporter 1 Gene Sequence Is Conserved in Insects and Encodes Proteins with Different Kinetic Properties Involved in Trehalose Import into Peripheral Tissues." *Insect Biochemistry and Molecular Biology* 40, no. 1 (January 1, 2010): 30–37. <https://doi.org/10.1016/j.ibmb.2009.12.006>.
 31. Moon, Randall T., Bruce Bowerman, Michael Boutros, and Norbert Perrimon. "The Promise and Perils of Wnt Signaling Through β -Catenin." *Science* 296, no. 5573 (May 31, 2002): 1644. <https://doi.org/10.1126/science.1071549>.
 32. Swarup, Sharan, and Esther M. Verheyen. "Wnt/Wingless Signaling in Drosophila." *Cold Spring Harbor Perspectives in Biology* 4, no. 6 (June 2012). <https://doi.org/10.1101/cshperspect.a007930>.
 33. Kijimoto, Teiya, and Armin P. Moczek. "Hedgehog Signaling Enables Nutrition-Responsive Inhibition of an Alternative Morph in a Polyphenic Beetle." *Proceedings of the National Academy of Sciences* 113, no. 21 (May 24, 2016): 5982–87. <https://doi.org/10.1073/pnas.1601505113>.
 34. Huelsken, Joerg, and Juergen Behrens. "The Wnt Signalling Pathway." *Journal of Cell Science* 115, no. 21 (November 1, 2002): 3977. <https://doi.org/10.1242/jcs.00089>.
 35. Seto, Elaine S., Hugo J. Bellen, and Thomas E. Lloyd. "When Cell Biology Meets Development: Endocytic Regulation of Signaling Pathways." *Genes & Development* 16, no. 11 (June 1, 2002): 1314–36. <https://doi.org/10.1101/gad.989602>.
 36. "Arrow Targets Wingless for Degradation." *Development* 132, no. 24 (December 15, 2005): e2404.
 37. Piddini, Eugenia, Francis Marshall, Laurence Dubois, Elizabeth Hirst, and Jean-Paul Vincent. "Arrow (LRP6) and Frizzled2 Cooperate to Degrade Wingless in Drosophila Imaginal Discs." *Development* 132, no. 24 (December 15, 2005): 5479–89. <https://doi.org/10.1242/dev.02145>.
 38. Baena-Lopez, Luis Alberto, Xavier Franch-Marro, and Jean-Paul Vincent. "Wingless Promotes Proliferative Growth in a Gradient-Independent Manner." *Science Signaling* 2, no. 91 (October 6, 2009): ra60–ra60. <https://doi.org/10.1126/scisignal.2000360>.
 39. Beermann, Anke, Romy Prühs, Rebekka Lutz, and Reinhard Schröder. "A Context-Dependent Combination of Wnt Receptors Controls Axis Elongation and Leg Development in a Short Germ Insect." *Development* 138, no. 13 (July 1, 2011): 2793. <https://doi.org/10.1242/dev.063644>.
 40. Wehrli, M., S. T. Dougan, K. Caldwell, L. O'Keefe, S. Schwartz, D. Vaizel-Ohayon, E. Schejter, A. Tomlinson, and S. DiNardo. "Arrow Encodes an LDL-Receptor-Related Protein Essential for Wingless Signalling." *Nature* 407, no. 6803 (September 28, 2000): 527–30. <https://doi.org/10.1038/35035110>.
 41. i5K Consortium. "The i5K Initiative: Advancing Arthropod Genomics for Knowledge, Human Health, Agriculture, and the Environment." *Journal of Heredity* 104, no. 5 (September 1, 2013): 595–600. <https://doi.org/10.1093/jhered/est050>.
 42. Schneider, Caroline A, Wayne S Rasband, and Kevin W Eliceiri. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nature Methods* 9, no. 7 (July 1, 2012): 671–75. <https://doi.org/10.1038/nmeth.2089>.
 43. Eberhard, William G., and Edgar E. Gutiérrez. "MALE DIMORPHISMS IN BEETLES AND EARWIGS AND THE QUESTION OF DEVELOPMENTAL CONSTRAINTS." *Evolution* 45, no. 1 (1991): 18–28.

44. Moczek AP. "Proximate Determination of Male Horn Dimorphism in the Beetle *Ontophagus Taurus* (Coleoptera: Scarabaeidae)." *Journal of Evolutionary Biology* 12, no. 1 (1999): 27.
<https://doi.org/10.1111/j.1558-5646.1991.tb05262.x>.
45. Marden, John I. "Use of Nested Orthogonal Contrasts in Analyzing Rank Data." *Journal of the American Statistical Association* 87, no. 418 (June 1, 1992): 307–18.
<https://doi.org/10.1080/01621459.1992.10475210>.
46. Stephanie Glen. "About / Contact" From StatisticsHowTo.com: Elementary Statistics for the rest of us! <https://www.statisticshowto.com/contact/>